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(54) Title: IMMUNOSUPPRESSIVE COMPOUNDS AND METHODS**(57) Abstract**

Compounds and methods for use in immunosuppressive and anti-inflammatory treatment, and for inhibiting male fertility; are described. The compounds are triptolide analogs with improved water solubility and low toxicity.

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IMMUNOSUPPRESSIVE COMPOUNDS AND METHODS

Field of the Invention

The present invention relates to compounds and methods for use in immunosuppressive and anti-inflammatory treatment, and for reducing male fertility.

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Background of the Invention

10 The immune system functions as the body's major defense against diseases caused by invading organisms. This complex system fights disease by killing invaders such as bacteria, viruses, parasites or cancerous cells while leaving the body's normal tissues unharmed. The immune system's ability to distinguish the body's normal tissues, or self, from foreign or cancerous tissue, or non-self, is an essential feature of normal immune system function. A second essential
15 feature is memory, the ability to remember a particular foreign invader and to mount an enhanced defensive response when the previously encountered invader returns. The loss of recognition of a particular tissue as self and the subsequent immune response directed against that tissue produce serious illness.

20 An autoimmune disease results from the immune system attacking the body's own organs or tissues, producing a clinical condition associated with the destruction of that tissue. An autoimmune attack directed against the joint lining tissue results in rheumatoid arthritis; an attack against the conducting fibers of the nervous system results in multiple sclerosis. The autoimmune diseases most likely share a common pathogenesis and the need for safe and effective therapy.

Rheumatoid arthritis is one of the most common of the autoimmune diseases. Current
25 treatments utilize three general classes of drugs (Schumacher, 1988): antiinflammatory agents (aspirin, non-steroidal antiinflammatory drugs and low dose corticosteroids); disease-modifying antirheumatic drugs, known as "DMARDs" (antimalarials, gold salts, penicillamine, and sulfasalazine) and immunosuppressive agents (azathioprine, chlorambucil, high dose corticosteroids, cyclophosphamide, methotrexate, nitrogen mustard, 6-mercaptopurine, vincristine, hydroxyurea,
30 and cyclosporin A). None of the available drugs are completely effective, and most are limited by severe toxicity.

In addition to their use in treating autoimmune conditions, immunosuppressive agents have also been used in treating or preventing transplantation rejection. Organ transplantation involving

human organ donors and human recipients (allografts), and non-human primate donors and human recipients (xenografts), has received considerable medical and scientific attention (Roberts, 1989; Platt, 1990; Keown, 1991; Wang and Morris, 1991; Hasan, 1992; Murase, 1993). To a great extent, these efforts have been aimed at eliminating, or at least reducing, the problem of rejection of the transplanted organ. In the absence of adequate immunosuppressive therapy, the transplanted organ is destroyed by the host immune system.

Another obstacle in transplantation, which has limited bone marrow transplants (BMT) in particular, is graft-versus-host disease (GVHD). GVHD is a condition in which transplanted marrow cells attack the recipient's cells (Thomas, 1975; Storb, 1984). Many BMT patients receiving HLA-identical marrow that tests negative in the mixed lymphocyte reaction (MLR) still develop GVHD, presumably because of a disparity between the recipient and donor at polymorphic non-HLA determinants. A large proportion of GVHD-afflicted individuals die as a result of GVHD (Weiden *et al.*, 1980).

Presently, the most commonly used agents for preventing transplant rejection include corticosteroids, antimetabolite drugs that reduce lymphocyte proliferation by inhibiting DNA and RNA synthesis such as azathioprine, immunosuppressive drugs such as cyclosporin A, which specifically inhibits T cell activation, and specific antibodies directed against T lymphocytes or surface receptors that mediate their activation (Briggs, 1991; Kennedy, 1983; Storb, 1985; Storb *et al.*, 1986). All of these drug therapies are limited in effectiveness, in part because the doses needed for effective treatment of transplant rejection may increase the patient's susceptibility to infection by a variety of opportunistic invaders, and in part because of direct toxicity and other side effects. For example, cyclosporin A, currently the most commonly used agent, is significantly toxic to the kidney. This nephrotoxicity limits the quantity of drug that can be safely given.

Recently, a number of compounds from the Chinese medicinal plant *Tripterygium Wilfordii* (TW) have been identified as having immunosuppressive activity. Representative compounds which have been isolated from TW include triptolide, 16-hydroxytriptolide, triptophenolide, triptidiolide, and celastrol, as described for example in Lipsky *et al.* (1994) and Zheng *et al.* (1991; 1994). However, the administration and therapeutic effectiveness of these compounds have been limited by their low water solubility.

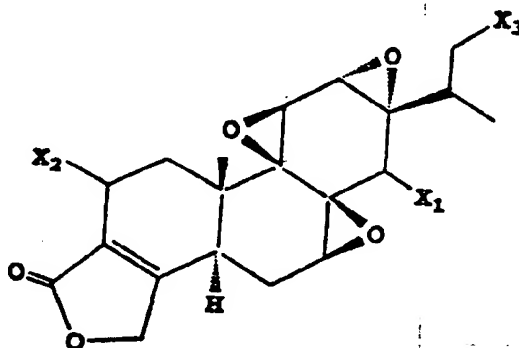
One approach to improving the effectiveness of these compounds is to formulate them in mixtures of ethanol and polyethoxylated castor oil (e.g., "CREMOPHOR EL"), allowing subsequent dilution in saline for intravenous administration. However, such formulations have suffered from high toxicity, due to the high concentration of solubilizing agent required to dissolve these compounds. For example, the ratio of solubilizing agent (ethanol plus "CREMOPHOR EL") to

triprolide in such formulations is typically on the order of 1000:1 or greater, due to the poor solubility of triprolone (Morris, 1991; Morris *et al.*, 1991). Standardization of dosage amounts is also more problematic with a suspension than with a solution.

It would therefore be desirable to provide immunosuppressive compounds having improved water solubility and low toxicity. In addition, it would be desirable for such compounds to exhibit immunosuppressive activity in their water soluble form, or to be convertible to an immunosuppressive form by metabolic processes *in vivo*. It would further be desirable to provide compounds having improved water solubilities that are useful as antifertility agents.

Summary of the Invention

The invention includes, in one aspect, a compound having the structure represented by Formula 1 below,



1

wherein X^1 is OH or OR^1 , and X^2 and X^3 are independently OH, OR^1 or H, with the proviso that at least one of X^1 , X^2 and X^3 is OR^1 , and at least one of X^2 and X^3 is H; and

R^1 is $-C(O)-Y-Z$, wherein

Y is a branched or unbranched C_1-C_6 alkyl or alkenyl chain; and

Z is $COOR^2$, NR^3R^3 , or $+NR^4R^4R^4$, where R^2 is a cation; R^3 and R^3 are independently H or branched or unbranched C_1-C_6 alkyl, hydroxyalkyl, or alkoxyalkyl, or R^3 and R^3 taken together form a 5- to 7-member heterocyclic ring whose ring atoms are selected from the group consisting of carbon, nitrogen, oxygen and sulfur, wherein the ring atoms include 2 to 6 carbon atoms, one or more nitrogen atoms, and optionally one or more oxygen or sulfur atoms, and wherein the ring is unsubstituted or is substituted with one or more groups selected from R^5 , OR^5 , NR^5R^6 , SR^5 , NO_2 , CN , $C(O)R^5$, $C(O)NR^5R^6$, $OC(O)R^5$, $OC(O)NR^5R^6$, and halogen (fluoro, chloro, bromo, or

iodo), where R^5 and R^6 are independently hydrogen, lower alkyl or lower alkenyl; and R^4 , $R^{4'}$, and $R^{4''}$ are independently branched or unbranched C_1 - C_6 alkyl, hydroxyalkyl or alkoxyalkyl.

In one general embodiment, the compound is a derivative of triptolide, wherein X_1 is OH or OR_1 as defined above, and X^2 and X^3 are H. In a second general embodiment, the compound is
5 a derivative of 16-hydroxyl triptolide, wherein X_1 and X_3 are OH or OR_1 , and X_2 is H. In a third general embodiment, the compound is a derivative of triptolide (2-hydroxytriptolide), wherein X_1 and X_2 are OH or OR_1 , and X^3 is H.

In one preferred embodiment, Z is $COOH$ or $COOR^2$, where R^2 is a metal ion, preferably Na^+ or K^+ . In an alternative embodiment, R^2 is a positively charged amine, preferably lysine,
10 triethylamine, or tris(hydroxymethyl)aminomethane. Preferably, R^2 is Na^+ , tris(hydroxymethyl)-aminomethane or lysine, and Y is a C_1 - C_4 alkyl chain.

In another preferred embodiment, Z is $NR^3R^{3'}$, where R^3 and $R^{3'}$ are independently H or branched or unbranched C_1 - C_6 alkyl, or together form a 5- to 7-member heterocyclic ring containing 2 to 6 carbon atoms, one or more nitrogen atoms, and optionally one or more oxygen
15 or sulfur atoms. Preferably, Z is dimethylamino, diethylamino, or N-morpholino, and Y is a C_1 - C_4 alkyl chain.

Where Z is a quaternary or protonated tertiary amino group, the compound also includes an anionic counterion. The anionic counterion is preferably a halide or a carboxylate-, sulfonate-, or sulfate-containing ion. More preferably, the counterion is chloride, bromide, acetate, oxalate,
20 maleate, fumarate, methanesulfonate, or toluenesulfonate.

In another aspect, the invention includes a method of effecting immunosuppression in a subject, wherein a composition as described above is administered to a subject in need of such treatment. The method is useful for inhibiting allograft rejection, xenograft rejection, and graft-versus-host disease, and in treating autoimmune diseases such as rheumatoid arthritis.

25 The compositions and method of the invention are also useful for the treatment of asthma, both intrinsic and extrinsic manifestations. For treatment of asthma, the composition is preferably administered via inhalation. The composition and method may also be used for treatment of other inflammatory conditions, such as traumatic inflammation, including traumatic inflammation accompanying head or neck injury.

30 The invention also provides a method of reducing male fertility in a male mammal, particularly humans, by administering to the mammal a compound in accordance with Formula 1 above, in an amount effective to inhibit the mammal's fertility.

In other aspects, the invention includes pharmaceutical compositions and medicaments for immunosuppressive treatment, antiinflammatory treatment, and for reducing male fertility. Such

compositions include a compound in accordance with Formula 1 above, in a pharmaceutically acceptable vehicle. In preferred embodiments, the vehicle is an aqueous carrier.

These and other objects and features of the invention will become more fully apparent when the following detailed description of the invention is read in conjunction with the accompanying
5 drawings.

Brief Description of the Drawings

Fig. 1 shows a scheme for preparing a carboxylated triptolide compound in accordance with the invention;

Fig. 2 shows a scheme for preparing amino derivatives of triptolide in accordance with the
10 invention;

Fig. 3 shows a scheme for preparing mono- and di-aminoester derivatives of 16-hydroxytriptolide;

Fig. 4 shows a scheme for preparing a 14-aminoester derivative of 16-hydroxytriptolide by means of protection and deprotection of the 16-hydroxyl group;

Fig. 5A shows a plot of allograft transplant survival time for untreated animals (closed squares), and animals treated with two different amounts of triptolide succinate tris(hydroxymethyl)aminomethane salt (YM-273) (open and closed circles);
15

Fig. 5B shows a plot of allograft transplant survival time for untreated animals (closed squares), and animals treated with two different amounts of triptolide succinate sodium salt (YM-274) (open and closed triangles);
20

Fig. 6 shows a plot of allograft transplant survival time for untreated animals (closed squares), and for animals treated with triptolide (T10, open squares), triptolide succinate tris(hydroxymethyl)aminomethane salt (YM-273, closed circles), and triptolide succinate sodium salt (YM-274, closed triangles);

Fig. 7 shows the effect of treating male mice with varying doses of triptolide succinate (YM-272) on testis weight (middle and right-hand column), compared with untreated mice (left-hand column), showing the mean (shaded regions) and standard error of the mean (SE, hollow regions) for groups of 5-6 mice; and
25

Fig. 8 shows the effect of treating male mice with varying doses of triptolide succinate (YM-272) on the time elapsed from onset of cohabitation to first birth of offspring, compared with untreated mice, showing the mean and standard error of the mean (SE) for groups of 5 mice.
30

Detailed Description of the Invention

I. Definitions

The terms below have the following meanings unless indicated otherwise.

"Triptolide derivatives" or "triptolide analogs" refers to derivatives of triptolide, 16-hydroxytriptolide and triptiolide (2-hydroxytriptolide) which are derivatized at one or more hydroxyl groups as described above.

"Alkyl" refers to a fully saturated monovalent or divalent radical containing carbon and hydrogen, and which may be cyclic, branched or a straight (unbranched) chain. Examples of alkyl groups are methyl, ethyl, n-butyl, n-heptyl, isopropyl, 2-methylpropyl, cyclopropyl, cyclopropylmethyl, cyclobutyl, cyclopentyl, cyclopentylethyl, and cyclohexyl.

"Lower alkyl" refers to an alkyl radical of one to four carbon atoms, as exemplified by methyl, ethyl, n-propyl, i-propyl, n-butyl, i-butyl, and t-butyl.

"Alkenyl" refers to a monovalent or divalent unsaturated, preferably mono-unsaturated, radical containing carbon and hydrogen, and which may be cyclic, branched or a straight chain.

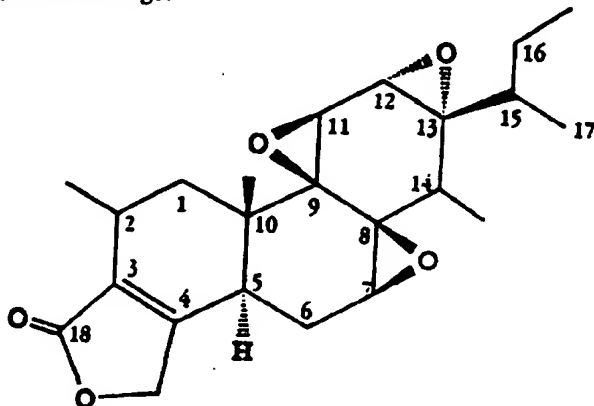
"Lower alkenyl" refers to such a radical having one to four carbon atoms.

A "5- to 7-member heterocyclic ring whose ring atoms are selected from the group consisting of carbon, nitrogen, oxygen and sulfur, wherein the ring atoms include 2 to 6 carbon atoms and one or more nitrogen atoms" refers to a heterocyclic ring whose ring atoms include one or more nitrogen atoms and, optionally, one or more oxygen or sulfur atoms. Examples are piperidine, piperazine, morpholine, pyrrolidine, thiomorpholine, and imidazole.

"Alkoxyalkyl" refers to an alkyl group as defined above, additionally containing an alkoxy substituent. Preferably, the alkyl portion of the alkoxy substituent is a lower alkyl group.

The term "mammal" is intended to have its ordinary meaning, and includes humans, dogs, cats, cows, sheep, mice, rats, and the like.

For the purposes of the current disclosure, the following numbering scheme is used for triptolide and triptolide analogs:



II. Synthesis of Triptolide Analogs

This section describes the synthesis of compounds in accordance with the present invention as defined by Formula 1 above. In general, the compounds are ester derivatives of triptolide, triptiolide or 16-hydroxytriptolide, wherein the attached ester substituents include one or more amino or carboxylate groups. The compounds possess greater water solubility than do the non-derivatized starting compounds and are useful as prodrugs for immunosuppressive, anti-inflammatory, and male antifertility applications.

The compounds of the invention may be prepared from triptolide, triptiolide, or 16-hydroxytriptolide obtained from the root xylem of the Chinese medicinal plant *Tripterygium Wilfordii* (TW) or from other known sources. The TW plant is found in the Fujian Province and other southern provinces of China; TW plant material can generally be obtained in China or through commercial sources in the United States. Methods for preparing triptolide, triptiolide, and 16-hydroxytriptolide are known in the art and are described, for example, in Kupchan *et al.* (1972); Kupchan *et al.* (1977); Lipsky *et al.* (1994); Pu *et al.* (1990); and Ma *et al.* (1992).

Synthetic schemes for preparing carboxylated derivatives of triptolide in accordance with the invention are shown in Fig. 1. With reference to the upper reaction path shown in the figure, triptolide (1) is reacted with an excess of a dicarboxylic acid of the form $\text{HO}_2\text{C}(\text{CH}_2)_m\text{CO}_2\text{H}$, where m is 1 to 4, in the presence of a coupling agent such as dicyclohexylcarbodiimide (DCC) and a catalytic amount of an acylation catalyst such as 4-(dimethylamino)pyridine (DMAP). The reaction conditions are effective to activate one or both carboxylate groups in the dicarboxylic acid towards reaction with the 14-hydroxyl group of (1), such that ester product (2) is formed. Any residual DCC attached to the free carboxyl in (2) may be released by addition of water, preferably under basic conditions.

A second method for preparing carboxylated derivatives of (1) is shown in the lower reaction path in Fig. 1. In this approach, (1) is reacted with a selected dicarboxylic acid anhydride, under conditions effective for the 14-hydroxyl group of (1) to attack one of the anhydride carbonyl groups to produce product (2). Exemplary conditions for this approach can be found in Example 1.

More generally, the methods illustrated in Fig. 1 can be used to prepare triptolide derivatives in accordance with Formula 1 above, wherein X_2 and X_3 are H and X_1 is $-\text{C}(\text{O})-\text{Y}-\text{Z}$ (*i.e.*, R_1 in Formula 1), wherein Y is a branched or unbranched C_1 - C_6 alkyl or alkenyl chain, and Z is COOR^2 , where R^2 is a cation.

Fig. 2 illustrates a method for preparing aminoester derivatives of triptolide. Triptolide (1) is reacted with an amine-substituted carboxylic acid, $\text{R}_\text{N}\text{CO}_2\text{H}$, in the presence of a coupling agent (*e.g.* DCC) and an acylation catalyst (*e.g.* DMAP). These reaction conditions may be used to pre-

pare a number of amino-derivatives, depending on the amino acid starting material. For example, reaction of (1) with N,N-dimethylglycine affords ester product (3a), as shown in Fig. 2 and described in Example 5. Similarly, reaction of (1) with 3-(N,N-diethylamino)propionic acid, 4-pyrrolidinobutyric acid, or 5-morpholinopentanoic acid affords product (3b), (3c), or (3d), respectively, as detailed in Examples 7-9. Amine salts in accordance with the invention are readily prepared by treatment with a selected acid, as in Example 6, or by using an ammonium salt form of R_NCO_2H in the coupling step, as in Examples 7 and 8.

Thus, it will be seen that the approach in Fig. 2 may be used to prepare amino-derivatives in accordance with structure (3) in Fig. 2 by using the appropriate starting materials, wherein R_N has the form Y-Z as defined in the Summary of the invention, Y is a branched or unbranched C_1 - C_6 alkyl or alkenyl chain, Z is NR^3R^3 or $^+NR^4R^4R^{4''}$, R^3 and R^3 are independently H or branched or unbranched C_1 - C_6 alkyl, hydroxyalkyl, or alkoxyalkyl, or, taken together, form a 5- to 7-member heterocyclic ring containing 2 to 6 carbon atoms, one or more nitrogen atoms, and optionally one or more oxygen or sulfur atoms, and wherein the ring is unsubstituted or is substituted with one or more groups selected from R^5 , OR^5 , NR^5R^6 , SR^5 , NO_2 , CN , $C(O)R^5$, $C(O)NR^5R^6$, $OC(O)R^5$, $OC(O)NR^5R^6$, and halogen (fluoro, chloro, bromo, or iodo), where R^5 and R^6 are independently hydrogen, lower alkyl or lower alkenyl; and R^4 , R^4 , and $R^{4''}$ are independently branched or unbranched C_1 - C_6 alkyl, hydroxyalkyl or alkoxyalkyl. In the case where Z is NR^3R^3 where R^3 and R^3 together form a heterocyclic ring, preferred ring moieties include morpholine, piperidine, pyrrolidine, and piperazine.

Moreover, while Figs. 1 and 2 illustrate reaction schemes using triptolide as the starting material, it will be appreciated that similar synthetic reaction schemes can be used to prepare corresponding ester derivatives of 16-hydroxytriptolide and triptidiolide.

Fig. 3 illustrates synthetic approaches for preparing mono- and diester derivatives of 16-hydroxytriptolide (4), a compound which contains two free hydroxyl groups. As can be seen from the figure, compound (4) contains a hydroxyl group at the 14-position which is linked to a secondary carbon atom, and a second hydroxyl group at the 16-position which is linked to a primary carbon atom. Since the hydroxyl group at the 16-position is more reactive than the 14-hydroxyl group for steric reasons, mono- and diester derivatives can be selectively made using appropriate reaction conditions.

As shown in the upper reaction path of Fig. 3, reaction of (4) with a stoichiometric amount of a selected carboxylic acid yields monoester (5) derivatized at the 16-position, with the 14-hydroxyl group remaining free. Conversely, as shown in the lower reaction path, reaction of (4)

with an excess of the carboxylic acid is effective to derivatize both hydroxyl groups, affording diester (6).

Monoester derivatives of 16-hydroxytriptolide (4) at the 14-position, rather than the 16-position, can be prepared by the general approach shown in Fig. 4. This approach takes advantage of the greater reactivity of the 16-hydroxyl group towards electrophiles, whereby the 16-hydroxyl can be selectively protected with a protecting group (PRT), as shown in the first step of Fig. 4. The protected compound (7) is then reacted with a selected carboxylic acid (RCO_2H) to esterify the 14-hydroxyl group, forming compound (8). The protecting group is then removed in a deprotection, to yield the desired 14-monoester (9). Suitable hydroxyl protecting groups for the purposes of the protection/deprotection scheme in Fig. 4 are known, and are described, for example, by Kocienski (1994). One preferred protecting group is a benzyl ether, which may be removed by catalytic hydrogenation (Kocienski, 1994, p. 46). Alternatively, a *t*-butyldimethylsilyl ether may be used. This group can be removed by, *e.g.*, treatment with tetrabutyl ammonium fluoride (TBAF).

Selective single derivatization of triptolide (2-hydroxytriptolide) is more difficult because of the similar reactivities of the two secondary hydroxyls. Accordingly, the 2- and 14-monoesters may be prepared as a mixture either by (1) reacting triptolide with a comparable amount of carboxylic acid (*e.g.*, 1 to 3 equivalents) or (2) briefly reacting triptolide with excess carboxylic acid followed quickly by addition of excess alcohol (*e.g.*, ethanol) to quench the excess carboxylic acid. In either case, a mixture of mono- and diester forms can be obtained which may then be separated by standard chromatographic methods such as HPLC.

Metal salts and amine salts of the amino and carboxyl ester compounds of the invention are readily prepared by reaction or exchange with an appropriate counterion, as described in Examples 2-4 and 6. In the case of carboxyl ester compounds such as (2), suitable counterions include sodium and potassium ions, as well as organic amines such as mono, di, tri, or tetraalkyl amines wherein the alkyl groups are lower alkyl or alkoxy groups.

III. Stability of Triptolide Derivatives

Sodium triptolide succinate, designated YM-274, was dissolved in D_2O , and the aqueous solution was stored at room temperature. Proton NMR spectra were taken at intervals, as described in Example 10A, and showed the compound to be unchanged after three months in solution. After five months, some decomposition was observed.

The stability of triptolide succinate in blood serum was determined as detailed in Example 10B. In this study, a solution of triptolide succinate (YM-262, free acid) in DMSO was mixed

with rat serum and incubated at 37°C. The mixture was assayed periodically by thin layer chromatography (TLC) to follow the hydrolysis of the triptolide succinate over time. Within the first 3 to 5 minutes, most of the triptolide succinate remained ($R_f = 0.45$). After 15 minutes, the triptolide succinate spot was gone and a new spot corresponding to triptolide had appeared ($R_f = 0.60$). Finally, after 45 minutes, the triptolide spot had also disappeared, and only low R_f material (blood serum components and decomposition products) remained. These results indicate that the triptolide ester is hydrolyzed in serum to release free triptolide in less than an hour.

IV. Biological Activity

A number of succinate salt derivatives in accordance with the present invention were examined for immunosuppressive activity using several biological assays. The compounds tested were carboxylated esters of triptolide prepared by succinylation of triptolide followed by salt formation, as detailed in Examples 1 to 4, with the following designations: free acid, YM-262; tris(hydroxymethyl)aminomethane (tris) salt, YM-273; sodium salt, YM-274; and L-(+)-lysine salt, YM-276. Triptolide succinate (free acid, YM-262) was also tested for antifertility effects.

A. Inhibition of IL-1 Action

The ability of the above compounds to suppress the cell-proliferative effect of IL-1 β *in vitro* (O'Gara, 1990) was examined as described in Example 11. Mouse thymocytes in culture were stimulated with IL-1 β in the presence of phytohemagglutinin (PHA) and increasing concentrations of triptolide (control) and triptolide derivatives. The cells were cultured for 72 hours; and during the last 18 hours, incubated with tritiated thymidine. DNA synthesis was assessed by measuring incorporation of radiolabeled thymidine. The results expressed in terms of IC_{50} (concentration effective to cause 50% suppression of proliferation) are shown in Table I.

Table I: IL-1 Assay

<u>Compound</u>	<u>IC₅₀</u>		<u>Toxicity</u>
	<u>(ng/ml)</u>	<u>(nmol/ml)</u>	<u>(ng/ml)</u>
Triptolide	1.9	0.0053	> 12500
YM-262	181	0.393	> 12500
YM-273	462	0.8	12000
YM-274	181	0.376	> 12500
YM-276	144	0.238	> 12500

With reference to column 3 in Table I, triptolide showed an IC_{50} value of about 0.005 nmol/ml, indicating that the free (underivatized) compound is a potent inhibitor of IL-1 β action. The free acid and salt forms of the triptolide 14-succinyl ester showed IC_{50} values that were approximately 45- to 150-fold higher than that of free triptolide in this *in vitro* assay. All of the ester derivatives showed low cytotoxicity (Table II, column 4), as measured by MTT assay (Example 13).

B. Mixed Lymphocyte Reaction (MLR)

Inhibition of cell proliferation by the subject compounds was assayed in the mixed lymphocyte reaction (MLR) (Bradley, 1980; Mishell, 1980). Spleen cells from female C57BL/6 mice, the "responder" cells, were prepared and co-cultured alone or in the presence of varying concentrations of the test compounds, with irradiated spleen cells prepared from female Balb/C mice, the "stimulator" cells. Prior irradiation of the stimulator cells rendered them unable to proliferate. A sample of the responder cells was also irradiated for use as a control. The non-irradiated responder cells proliferate in the presence of the allogenic stimulator cells. After a 78-hour incubation, tritiated thymidine was added to the mixed cell cultures, and incorporation of the labeled nucleotide into DNA was measured as an index of cell proliferation. The results are shown in Table II.

Table II: MLR Assay

<u>Compound</u>	<u>IC_{50}</u>		<u>Toxicity</u>
	<u>(ng/ml)</u>	<u>(nmol/ml)</u>	<u>(ng/ml)</u>
Triptolide	0.9	0.0025	13.3
YM-262	86	0.187	2258
YM-273	180	0.31	5529
YM-274	90	0.187	2498
YM-276	63	0.104	1936

As can be seen, the data in this *in vitro* assay were qualitatively similar to those obtained with the IL-1 assay in Table I above, in that the free acid and salt forms of the triptolide 14-succinyl ester showed IC_{50} values that were approximately 40- to 125-fold higher than that of free triptolide. As shown in Table II, all of the ester derivatives showed low cytotoxicity, and much lower toxicity than free triptolide, as measured by MTT assay (Example 13).

C. Cardiac Allograft Survival

Treatment of transplantation rejection, in accordance with the invention, is illustrated for rejection of an allograft by the *in vivo* heart transplantation model used in Example 14. The method

involves a well-characterized rat model system (Ono and Lindsey, 1969) in which a transplanted heart is attached to the abdominal great vessels of an allogeneic recipient animal, and the viability of the transplanted heart is gauged by the heart's ability to beat in the recipient animal.

In one study, the animals were administered by intraperitoneal injections, from one day preceding to 14 days following heart transplantation, either control solution (5% ethanol, 10 ml/kg), triptolide (designated as T10), or with two concentrations each of YM-273 and YM-274. There were three animals in each group, except for the control group in which five animals were used. The results are shown in Figs. 5A and 5B.

As can be seen from Fig. 5A, YM-273 at a dosage level of 0.1 mg/kg (open circles) gave a mean survival time of 7 days, similar to the results obtained with the control group. However, a dosage of 0.4 mg/kg (closed circles) showed a substantial improvement over the control, with a mean survival time of 24 days.

With reference to Fig. 5B, YM-274 administered at a dosage level of 0.0837 mg/kg (open triangles) gave a mean survival time of 10 days, whereas a dosage of 0.33 mg/kg (closed triangles) gave a mean survival time of approximately 50 days.

Fig. 6 compares the immunosuppressive effect of the higher doses of YM-273 and YM-274, described above, with equivalent doses (on a molar basis) of triptolide (T-10). As can be seen, the mean survival time obtained following administration of succinate derivatives YM-273 (tris salt) was somewhat better than that seen with underivatized triptolide, and was substantially better (50 \pm 7 days) in the case of succinate derivative YM-274 (sodium salt).

The above results indicate that the water soluble ester compounds of the invention have significant immunosuppressive activity *in vivo*. As described in Section III, above, triptolide succinate was hydrolyzed in blood serum to triptolide within about 15 minutes. It is likely that the compounds of the invention act as prodrugs, in that the ester groups are cleaved *in vivo* to produce the more active, underivatized triptolide compound.

D. Inhibition of Male Fertility

The compounds of the invention are also effective in reducing or inhibiting male fertility, wherein administration of a compound of the invention to a male mammal is effective to reduce the potency of the male's semen, thereby reducing or blocking fertilization. Triptolide succinate (YM-262) was tested for inhibition of fertility in male BDF1 mice, according to protocols described in Example 15. The compound was administered intraperitoneally (IP) in a saline solution, using a cycle of daily administration for 5 days followed by a two day cessation of treatment. Dose levels were 0.04 or 0.13 mg/kg/day. The cycle was repeated for 5 weeks before evaluation of physiological effects (testis weight) and reproductive performance.

As shown in Fig. 7, administration of 0.13 mg/kg/day of YM-262, according to the above schedule, produced a reduction in testis weight of approximately 13% in comparison to the saline-treated control group.

5 The effect of the compound on fertility was tested with groups of five male mice treated according to the same schedule. Each male was housed with two females starting on day 32, after termination of YM-262 treatment.

10 The time from beginning of cohabitation to birth of the litters is shown in Fig. 8. The saline-treated (control) group produced the first litter after a mean of 24 days, four days longer than the expected gestation period (about 20 days) as found for the saline control group. The value for the group receiving YM-262 at 0.04 mg/kg/day was 28 days (a 17% delay), indicating an approximately four-day period of unproductive mating. For the group receiving YM-262 at 0.13 mg/kg/day, the time from cohabitation to birth was 45 days, an 88% increase in gestation time compared to the control group.

15 These results indicate that YM-262 treatment at 0.13 mg/kg/day over a period of 32 days delayed the recovery of fertility, after termination of therapy, by 21 days. Furthermore, the antifertility effect was reversible, since cessation of administration of the drug was followed by successful pregnancies. It will be appreciated that longer periods of male infertility can be achieved by continued administration of triptolide compounds in accordance with the present invention. The compounds may be administered with other antifertility agents for improved effects.

20 V. Therapeutic Compositions

Formulations containing the triptolide analogs of the invention may take the form of solid, semi-solid, lyophilized powder, or liquid dosage forms, such as, for example, tablets, pills, capsules, powders, sustained-release formulations, solutions, suspensions, emulsions, suppositories, retention enemas, creams, ointments, lotions, aerosols or the like, preferably in unit dosage forms
25 suitable for simple administration of precise dosages.

The compositions typically include a conventional pharmaceutical carrier or excipient and may additionally include other medicinal agents, carriers, adjuvants, and the like. Preferably, the composition will be about 0.5% to 75% by weight of a compound or compounds of the invention, with the remainder consisting of suitable pharmaceutical excipients. For oral administration, such
30 excipients include pharmaceutical grades of mannitol, lactose, starch, magnesium stearate, sodium saccharine, talcum, cellulose, glucose, gelatin, sucrose, magnesium carbonate, and the like. If desired, the composition may also contain minor amounts of non-toxic auxiliary substances such as wetting agents, emulsifying agents, or buffers.

Liquid compositions can be prepared by dissolving or dispersing the triptolide analog (about 0.5% to about 20%), and optional pharmaceutical adjuvants, in a carrier, such as, for example, aqueous saline, aqueous dextrose, glycerol, or ethanol, to form a solution or suspension.

The composition may be administered to a subject orally, transdermally or parenterally, *e.g.*,
5 by intravenous, subcutaneous, intraperitoneal, or intramuscular injection. For use in oral liquid preparation, the composition may be prepared as a solution, suspension, emulsion, or syrup, being supplied either in liquid form or a dried form suitable for hydration in water or normal saline. For parenteral administration, an injectable composition for parenteral administration will typically contain the triptolide analog in a suitable intravenous solution, such as sterile physiological salt
10 solution. When the composition is employed in the form of solid preparations for oral administration, the preparations may be tablets, granules, powders, capsules or the like. In a tablet formulation, the composition is typically formulated with additives; *e.g.* an excipient such as a saccharide or cellulose preparation, a binder such as starch paste or methyl cellulose, a filler, a disintegrator, and other additives typically usually used in the manufacture of medical prepara-
15 tions.

The high water solubility of the compounds of the invention make them particularly advantageous for administering in aqueous solution, *e.g.* by intraperitoneal injection. Dissolution studies conducted in support of the present invention have shown that the compounds of the present invention readily dissolve in aqueous solution when the compounds are prepared in
20 powdered form. Thus, the compounds are particularly suitable for tablet or capsule formulations. Composition or medicaments in accordance with the invention may also be formulated as a suspension in a lipid (*e.g.*, a triglyceride or a polyethoxylated castor oil such as "CREMOPHOR EL") or phospholipid, in a liposomal suspension, or in an aqueous emulsion.

The compound may also be administered by inhalation, in the form of aerosol particles,
25 either solid or liquid, preferably of respirable size. Such particles are sufficiently small to pass through the mouth and larynx upon inhalation and into the bronchi and alveoli of the lungs. In general, particles ranging from about 1 to 10 microns in size, and preferably less than about 5 microns in size, are respirable.

Compositions containing respirable dry particles of micronized active agent may be prepared
30 by grinding dry active agent and passing the micronized composition through a 400 mesh screen to break up or separate out large agglomerates. The solid particulate form of the active agent may contain a dispersant to facilitate the formation of an aerosol. A suitable dispersant is lactose, which may be blended with the active agent in any suitable ratio (*e.g.*, a 1:1 ratio by weight).

Any solid particulate medicament aerosol generator may be used to administer the solid particles. Such generators, such as the DeVilbiss nebulizer (DeVilbiss Co., Somerset, Pa.), produce particles which are respirable, as explained above, and generate a volume of aerosol containing a predetermined metered dose of a medicament at a rate suitable for human administration. Liquid compositions for inhalation comprise the active agent dispersed in an aqueous carrier, such as sterile pyrogen free saline solution or sterile pyrogen free water. If desired, the composition may be mixed with a propellant to assist in spraying the composition and forming an aerosol.

Methods for preparing such dosage forms are known or will be apparent to those skilled in the art; for example, see Remington's Pharmaceutical Sciences (1980). The composition to be administered will contain a quantity of the selected compound in a pharmaceutically effective amount for effecting immunosuppression, or reduction of fertility, in a subject.

V. Treatment Method

The compositions of the present invention may be employed in immunosuppression therapy, in particular, therapy in treating an autoimmune disease, graft-versus-host disease (GVHD), or transplantation rejection, particularly allograft rejection or xenograft rejection. The compositions are also useful for inhibiting male fertility, for treatment of both intrinsic and extrinsic forms of asthma, and for treatment of other inflammatory conditions, such as traumatic inflammation.

Table III below gives a list of autoimmune diseases which are appropriate for immunotherapy.

Table III

Autoimmune Diseases	
Disease	Tissue Affected
Addison's disease	adrenal
Allergies	inflammatory cells
Asthma	bronchi
Atherosclerosis	vessel walls
Crohn's disease	intestine
Diabetes (Type I)	pancreas
Graves' disease	thyroid
Guillain-Barré Syndrome	nerve cells
Inflammatory bowel disease	intestine

Autoimmune Diseases	
Disease	Tissue Affected
Systemic Lupus erythematosus (SLE)	multiple tissues
Multiple sclerosis	nerve cells
Myasthenia Gravis	neuromuscular junction
Psoriasis	skin
Primary biliary cirrhosis	liver
Rheumatoid arthritis	joint lining
Uveitis	eye

5

In treating an autoimmune condition, the patient is given the composition on a periodic basis, *e.g.*, 1-2 times per week at a dosage level sufficient to reduce symptoms and improve patient comfort.

10

For treating rheumatoid arthritis, the composition may be administered by intravenous injection or by direct injection into the affected joint. The patient may be treated at repeated intervals of at least 24 hours, over a several week period following the onset of symptoms of the disease in the patient.

15

For the treatment of systemic lupus erythematosus (SLE), as another example, the composition may be administered by oral or parenteral administration, such as intravenous (IV) administration.

20

The dose that is administered is preferably in the range 1 to 25 mg/kg patient body weight per day, with lower amounts being preferred for parenteral administration, and higher amounts being preferred for oral administration. Optimum dosages can be determined by routine experimentation according to methods known in the art.

25

For therapy in transplantation rejection, such as allograft or xenograft rejection, the method is intended particularly for the treatment of rejection of heart, kidney, liver, cellular, and bone marrow transplants. The method may also be used in the treatment of graft-versus-host disease (GVHD), in which transplanted immune cells attack the allogeneic host. Initial treatment is administered perioperatively. In addition, the composition may be administered chronically to prevent graft rejection, or in treating acute episodes of late graft rejection. As above, the dose administered is preferably 1 to 25 mg/kg patient body weight per day, with lower amounts being preferred for parenteral administration, and higher amounts for oral administration. The dose may

be increased or decreased appropriately, depending on the response of the patient, and over the period of treatment, the ability of the patient to resist infection.

The treatment is typically initiated perioperatively, either soon before or soon after the surgical transplantation procedure, and is continued on a daily dosing regimen, for a period of at least several weeks, for treatment of acute transplantation rejection. During the treatment period, the patient may be tested periodically for immunosuppression level, *e.g.*, by a mixed lymphocyte reaction involving allogenic lymphocytes, or by taking a biopsy of the transplanted tissue.

In another aspect, for immunosuppressive treatments as discussed above, the invention includes a method of suppressing allograft rejection, xenograft rejection, or graft versus host disease in a host subject wherein a compound of the present invention is administered concurrently with another immunosuppressive drug. The method includes administering to the subject, an immunosuppressant drug such as cyclosporin A, FK506, azathioprine, rapamycin, mycophenolic acid, or a glucocorticoid, in an amount that is substantially less than the dose needed to achieve effective suppression of allograft rejection, when the compound is administered alone. A potentiator, comprising a triptolide analog of formula 1, as described above, is administered in an amount effective to suppress allograft rejection, xenograft rejection, or GVHD in the host, when administered in combination with the immunosuppressive compound. By "an amount that is substantially less than the dose needed to achieve effective suppression of allograft rejection (or xenograft rejection, or rejection due to GVHD) when the compound is administered alone" is meant an amount of immunosuppressant drug which is below 50%, and preferably less than 33%, of the amount that would otherwise be administered if used without a triptolide analog of the invention. Allograft or xenograft rejection is "effectively suppressed" or "suppressed" in a host if the survival time of the transplant or graft in the host is extended by a statistically meaningful period over the survival time in the host in the absence of immunosuppression therapy. Typically, an effective suppression of graft rejection is a period of at least or more weeks, and may be up to several months or more. Alternatively, the triptolide compound ("potentiator") and immunosuppressive drug are administered in amounts such that the resultant immunosuppression is greater than what would be expected or obtained from the sum of the effects obtained with the immunosuppressive drug and triptolide compound used alone.

The immunosuppressive drug and potentiator may both administered at regular intervals over a time period of at least 2 weeks, and may be administered either orally or parenterally. The

amount of immunosuppressant drug administered is typically between about 20% and 100% of the amount of drug needed to suppress rejection in the host.

In the potentiated immunosuppressant therapy method of the invention, a triptolide analog of Formula 1 above may be administered with an immunosuppressant drug together in the same formulation, or separately in separate formulations. Where separate formulations are used, the triptolide analog or compound and the immunosuppressant drug can be administered by different routes.

The immunosuppressant drug which is administered with the triptolide analog is preferably one of the following:

- 10 (a) Cyclosporin A or cyclosporin C ("cyclosporin"), a non-polar cyclic oligopeptide;
- (b) FK506, a fungal macrolide immunosuppressant; ✓
- ✓ (c) azathioprine, or 6-[(1-methyl-4-nitro-1H-imidazole-5yl)thio]1H-purine; ✓
- (d) methotrexate, ✓
- (e) rapamycin, a fungal macrolide immunosuppressant; ✓
- 15 (f) mycophenolic acid, or 6-(1,3-Dihydro-4-hydroxy-6-methoxy-7-methyl-3-oxy-5-isobenzofuranyl)-4-methyl-4-hexanoic acid; and ✓
- (g) an immunosuppressant glucocorticoid, such as prednisone or dexamethasone.

The proportions of the two components (triptolide analog and immunosuppressant drug) are preferably in the range of 1:50 to 50:1 by weight. The immunosuppressive compound is preferably cyclosporin A, administered in an amount less than 1/3 the usual suppressive dose.

For male fertility inhibition, the compound may be administered by intraperitoneal (IP) or intravenous injection, or, preferably, by oral administration, at a dosage and frequency effective to reduce or block the fertility of the subject. The useful dose varies as a function of the administration route. For human subjects, the dose will vary, for example, from 0.1 to 15 mg/kg per day in an adult subject when administered orally.

In the treatment of asthma, intranasal administration (drops or spray), inhalation of an aerosol through the mouth, or conventional oral administration is generally preferred. The active agent may also be applied to the nasal respiratory epithelium as a topically applied liquid medicament.

If the patient is unable to swallow, or oral absorption is otherwise impaired, the preferred systemic route of administration will be parenteral, intranasal, or topical.

The compounds of the invention may also be administered to an individual after onset of asthma to reduce breathing difficulty, or they may be administered prophylactically, that is, before the bronchospasm begins in an asthma attack, to prevent or minimize its occurrence.

The following examples are intended to illustrate but not in any way limit the invention.

Example 1

Triptolide Succinate (YM-262)

Triptolide (100 mg) in 10 ml of pyridine was treated with succinic anhydride (150 mg) at room temperature. The reaction was carried out at 85°C for 30 hours under a nitrogen atmosphere. Hexane (50 ml) was added to the resultant mixture to precipitate a crude product, which was collected by filtration and washed with hexane. The crude product was recrystallized from ether/hexane to yield 90 mg (70%) of triptolide succinate (YM-262), m.p. 111-113°C.

IR(KBr): 3431.8, 2974.6, 1743.8, 1375.5, 1159.4, 1022.4 cm⁻¹. ¹H NMR (CDCl₃): 5.08 (1H, s, 14-CH), 4.67 (2H, s, 19-CH₂), 3.82 (1H, d, 11-CH), 3.50 (1H, d, 12-CH), 3.43 (1H, d, 7-CH), 2.75 (5H, m, CH₂CH₂, 5-CH), 2.30 (1H, d-m, 15-CH), 2.15 (2H, m, 6-CH₂, 2-CH₂), 1.88 (2H, m, 2-CH₂, 6-CH₂B), 1.55 (1H, m, 1-CH₂), 1.20 (1H, m, 1-CH₂), 1.05 (3H, s, 20-CH₃), 0.95 (3H, d, 16-CH₃), 0.83 (3H, d, 17-CH₃) ppm. MS (m/z): 461 (M+1).

Example 2

Triptolide Succinate Tris Salt (YM-273)

Triptolide succinate (20 mg) was mixed with tris(hydroxymethyl)amino methane (5.3 mg) in 20 ml of water and stirred for one hour. The solution was filtered and the filtrate was lyophilized to yield 24 mg (96%) of white powder.

IR(KBr): 3391, 2937.96, 1745.80, 1562.9, 1411.67, 1159, 1066.57, 1024.57 cm⁻¹. ¹H NMR (D₆-DMSO, ppm): 5.00 (1H, s, 14-CH), 4.85 (2H, d, 19-CH₂), 3.95 (1H, d, 11-CH), 3.70 (1H, d, 12-CH), 3.55 (1H, d, 7-CH), 3.30 (6H, s, 3CH₂O), 2.65 (1H, m, 5H), 2.45 (2H, m, CH₂), 2.20 (3H, m, CH₂, 15-CH), 1.90 (4H, m, 6-CH₂, 2-CH₂), 1.34 (2H, br, 1-CH₂), 0.95 (3H, s, 20-CH₃), 0.88 (3H, d, 16-CH₃), 0.75 (3H, d, 17-CH₃).

Example 3

Triptolide Succinate Sodium Salt (YM-274)

Triptolide succinate (20 mg) was mixed with sodium bicarbonate (3.65 mg) in 20 ml of water and stirred for 30 min. The water solution was filtered and the filtrate was lyophilized to yield 20 mg (95%) white powder.

IR(KBr): 3431.8, 2975.56, 1743.87, 1577.97, 1419.79, 1163.22, 1022.40 cm^{-1} . ^1H NMR (D₆-DMSO, ppm): 5.00 (1H, s, 14-CH), 4.85 (2H, d, 19-CH₂), 3.95 (1H, d, 11-CH), 3.70 (1H, d, 12-CH), 3.55 (1H, d, 7-CH), 2.58 (1H, m, 5H), 2.45 (2H, m, CH₂), 2.20 (3H, m, CH₂, 15-CH), 1.90 (4H, m, 6-CH₂, 2-CH₂), 1.34 (2H, br, 1-CH₂), 0.95 (3H, s, 20-CH₃), 0.88 (3H, d, 16-CH₃), 0.75 (3H, d, 17-CH₃).

Example 4

Triptolide Succinate Lysine Salt (YM-276)

Triptolide succinate (20 mg) mixed with L-(+)-lysine (6.3 mg) in 20 ml water was stirred for one hour. The solution was filtered and the filtrate was lyophilized to yield 25 mg (95%) white powder.

IR(KBr): 3431.8, 2934.0, 1743.9, 1560.6, 1399.9, 1147.6, 1018.6 cm^{-1} . ^1H NMR (D₆-DMSO, ppm): 5.00 (1H, s, 14-CH), 4.85 (2H, d, 19-CH₂), 3.95 (1H, d, 11-CH), 3.78 (1H, d, 12-CH), 3.55 (1H, d, 7-CH), 3.50 (6H, br, 2NH₃), 3.15 (1H, m, -CH), 2.70 (1H, m, 5H), 2.65 (1H, m, CH₂), 2.4 (2H, m, CH₂), 2.20 (3H, m, CH₂, 15-CH), 1.90 (4H, m, 6-CH₂, 2-CH₂), 1.40 (6H, m, CH₂CH₂CH₂), 1.34 (2H, br, 1-CH₂), 0.95 (3H, s, 20-CH₃), 0.88 (3H, d, 16-CH₃), 0.75 (3H, d, 17-CH₃) ppm.

Example 5

Synthesis of 14-N,N-Dimethylglycinate Ester of Triptolide

Into a dry 100 mL round bottom flask is placed 1 eq. of triptolide and 2 eq. each of N,N-dimethyl glycine and DCC (dicyclohexylcarbodiimide). The flask is placed under a nitrogen atmosphere, and anhydrous CH₂Cl₂ (dried over P₂O₅) is added, followed by a catalytic amount of DMAP (4-dimethylaminopyridine). The solution is stirred overnight at room temperature. The reaction is worked up by filtering off the dicyclohexylurea, removing the solvent by evaporation, and chromatographing the obtained solid on silica gel.

Example 6

Synthesis of Methanesulfonic Acid Salt of 14-N,N-Dimethylglycinate Ester of Triptolide

Into a dry round bottom flask is placed 1 eq. of the 14-N,N-dimethylglycinate ester of triptolide, as prepared in Example 5. The compound is dissolved in anhydrous CH₂Cl₂ (distilled from P₂O₅), and to the resulting solution is added 1 eq. of a stock solution of methanesulfonic acid in diethyl ether. The solvent is immediately removed to yield a white solid.

Example 7

Synthesis of 14-(3-(N,N-Dimethylamino)propionate) Hydrochloric Salt Ester of Triptolide

Into a dry 100 mL round bottom flask is placed 1 eq. of triptolide and 2 eq. each of N,N-dimethylamino propionic acid and DCC (dicyclohexylcarbodiimide). The flask is placed under

a nitrogen atmosphere, and anhydrous CH_2Cl_2 (dried over P_2O_5) is added, followed by a catalytic amount of DMAP (4-dimethylaminopyridine). The solution is stirred overnight at room temperature. The dicyclohexylurea is filtered off, and the solvent is removed by evaporation. The crude product is then chromatographed on silica gel.

5

Example 8

Synthesis of 14-(4'-N-pyrrolidino)butyrate) Hydrochloride Salt Ester of Triptolide

Into a dry round bottom flask is placed 1 eq. of triptolide, 2 eq. of 4-pyrrolidinobutyric acid hydrochloride salt, and anhydrous CH_2Cl_2 (distilled from P_2O_5). The resulting solution is placed under a nitrogen atmosphere, and 2 eq. of DCC and a catalytic amount of DMAP is added. The solution is stirred overnight at room temperature. The reaction is worked up by filtering off the dicyclohexylurea, removing the solvent by evaporation, and chromatographing the obtained solid on silica gel.

10

Example 9

Synthesis of Bis N,N-Dimethylglycinate Ester of 16-Hydroxytriptolide

The title compound is synthesized by the reaction of 1 eq. of 16-hydroxytriptolide, 3 eq. of N,N-dimethylglycine, 3.3 eq. of DCC, and 0.16 eq. of DMAP in anhydrous CH_2Cl_2 , followed by working up as described in the previous example.

15

The bis-N,N-dimethylglycinate ester at the 2- and 14-positions of triptolide is prepared in a similar fashion from triptolide (2-hydroxytriptolide).

20

Example 10

Stability of Triptolide Succinate (YM-262)

A. Stability in Water

A solution of sodium triptolide succinate (YM-274) in D_2O was prepared at a concentration of 3 mg/ml and stored at room temperature. The solution was analyzed by ^1H NMR at intervals of 1, 3, 5, 15, 45, 90, 180 minutes; 1, 7, 14 days; and 1, 2, 3, and 5 months. There was no appreciable change in the NMR spectrum during the first three months. Some decomposition was apparent after 5 months.

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B. Stability in Blood Serum

A solution of triptolide succinate (free acid; YM-262) in DMSO was made at a concentration of 25 mg/ml, and 0.1 ml of this solution was mixed with 0.5 ml of rat serum. The mixture was incubated at 37°C . Aliquots of the mixture were taken at 1, 3, 5, 15, 45 minutes and 18 hours and analyzed by thin layer chromatography (TLC). The TLC plates were developed in 1:5 $\text{CH}_2\text{Cl}_2/\text{Et}_2\text{O}$. After development, the plates were treated with iodine vapor and examined

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under a UV lamp. Triptolide and triptolide succinate were used as reference compounds ($R_f = 0.60$ and 0.45 , respectively).

After 3 minutes, only triptolide succinate was detected by TLC. After 15 minutes, the triptolide succinate spot was gone ($R_f = 0.45$), and a new spot corresponding to triptolide appeared ($R_f = 0.60$). After 45 minutes, the triptolide spot also disappeared, and only low R_f material (blood serum components and decomposition products) remained.

Example 11

Inhibition of IL-1 Action on Murine Thymocytes

C3H/HeN mouse thymocytes were prepared and the action of IL-1 together with PHA, which stimulate proliferation of thymocyte, was measured using standard techniques (O'Gara, 1990; Mishell, 1980). Three- to six-week old C3H/HeN male mice (Simonson Laboratory, Gilroy, CA) were sacrificed by CO_2 inhalation. Thymi were removed, separated from adherent non-thymic tissue, homogenized in Hank's balanced salt solution (HBSS, Gibco) using a glass homogenizer, and centrifuged at $200 \times g$ for 10 minutes at 15°C . Following an additional wash in HBSS, the thymocytes were resuspended in RPMI 1640 medium containing $50 \mu\text{M}$ 2-mercaptoethanol, 2mM glutamine, 1mM sodium pyruvate, non-essential amino acids 100U/ml penicillin, $100 \mu\text{g/ml}$ streptomycin, and 10% heat-inactivated fetal bovine serum.

Cells were cultured in round-bottom 96 well microtiter tissue culture plates, 6×10^5 cells per well, in a volume of $100 \mu\text{l}$. Recombinant human IL- 1β (R & D Systems #201-LB) together with Phytohemagglutinin P (PHA, Pharmacia) were added to the cells in volume of $25 \mu\text{l}$ per well to achieve a final concentration of 0.08ng/ml and $10 \mu\text{g/ml}$, respectively. Samples were dissolved in DMSO (10mg/ml), then diluted in culture medium. Twenty five microliters of the test sample was added to each well to achieve the final compound concentrations for each experiment. Cells with PHA together with IL-1 served as controls. The total volume for each well was $150 \mu\text{l}$.

Plates were incubated at 37°C in a $5\% \text{CO}_2$ incubator for 72 hours. Fifty microliters of culture medium containing $0.5 \mu\text{Ci}$ (^3H)-thymidine (Amersham, 49Ci/mmol) was added to each well prior to the last 18 hours of incubation. Cells were then harvested and counted. The results were reported as counts per minute (cpm) per 6×10^5 cells.

The following formula was used to calculate the percent of suppression of IL-1 activity, and IC_{50} (concentration of sample yielding 50% suppression of proliferation) was used to indicate the suppressive activity of the sample.

$$\begin{aligned} \% \text{ suppression of IL-1 activity} = \\ (1 - \text{sample cpm} / (\text{IL-1} + \text{PHA control cpm})) \times 100 \end{aligned}$$

The results are shown in Table I above.

Example 12Mixed Lymphocyte Reaction (MLR) Assay

In this study, the responder cells (R) were spleen cells from female C57BL/6 mice, and the stimulator cells (S) were spleen cells from female Balb/C mice 6 to 8 weeks of age (Jackson, Bar Harbor, Maine). The spleens were aseptically removed from the mice and placed into 10 ml of cold HBSS in a sterile petri dish. The spleen was cut in half and gently pressed between the frosted ends of 2 sterile microslides. The cell suspension was then filtered through sterile nylon mesh (Nytex, Tetco #HD-3-85) into a 15 ml conical polypropylene centrifuge tube and centrifuged at $200 \times g$ for 10 minutes in a Beckman GPR tabletop centrifuge (GH-3.7 Rotor). Following an additional wash in HBSS, the spleen cells were resuspended in RPMI 1640 medium (Gibco) containing 50 μM 2-mercaptoethanol, 2 mM glutamine, 100 U/ml penicillin, 100 $\mu\text{g/ml}$ streptomycin and 10% heat-inactivated fetal bovine serum.

The stimulator cells (S) and part of the responder cells (R) were diluted at 10×10^6 cells/ml and irradiated at 20 cGy with a Cesium Irradiator (Department of Radiation Oncology, Stanford University, CA) to inhibit proliferation. The irradiated cells were washed once to remove any toxic free radicals and their products resulting from irradiation. The responder cells (R), irradiated stimulator cells (Sx) and irradiated responder cell (Rx) were all diluted to 4×10^6 cells/ml.

In the assay, 4×10^5 cells of R were cocultured with 4×10^5 cells of Sx in 200 μl of medium in round bottom 96 well tissue culture plates. Fifty micro-liters of test samples at various concentrations were added to the cells. The wells receiving no test samples would get the maximum proliferation. Several controls were used in the assay. The irradiated responder cells (Rx) were also added to the responder cells with and without the test samples. Rx or Sx alone were checked to make sure no proliferation occurred after irradiation. The spontaneous proliferation of R was also measured.

The culture plates were incubated at 37°C in a 5% CO_2 incubator for four days. The cells were labelled with 1 μCi of (^3H) -thymidine (Amersham, 49 Ci/mmol) in 20 μl of medium for the last 18 hours. Cells were then harvested and counted. The results were reported as counts per minute (cpm) per well. Percent of suppression and IC_{50} (concentration of sample producing 50% suppression of proliferation) were used to indicate the suppressive activity of the sample. Sample cpm was calculated as $(\text{R} + \text{Sx} + \text{sample})\text{cpm} - (\text{R} + \text{Rx} + \text{sample})\text{cpm}$; control cpm was calculated as $(\text{R} + \text{Sx})\text{cpm} - (\text{R} + \text{Rx})\text{cpm}$. Percent suppression of MLR activity was calculated as $(1 - \text{sample cpm}/\text{control cpm}) \times 100$. IC_{50} was determined from percent suppression to indicate the suppressive activity of the sample. The results are shown in Table II above.

Example 13

Evaluation of Cytotoxicity

Potential cytotoxicity of the test samples was assessed by the measurement of their effect on the reduction of MTT (3-[4,5-Dimethylthiazole-2-yl]-2,5-diphenyltetrazolium bromide) by cultured cells. MTT, a yellow-colored compound, is reduced by mitochondrial enzymes to form the purple crystalline reduction product formazane, providing an index of cellular respiration on viable cells as well as a sensitive assay for cytotoxicity (Mossmann, 1983).

The cytotoxicity was assessed in cultured human PBMC and mouse thymocytes. A stock solution of MTT (Sigma) at 5 mg/ml in phosphate buffered saline, pH 7.4, was prepared and stored in the dark at 4°C. PBMCs or thymocytes were cultured with various concentrations of test samples in flatbottom 96-well tissue culture plates (Costar) under the same conditions as those described above, but the stimulants (X-35 or IL-1 + PHA) were replaced by appropriate medium. Untreated cells with medium alone and without the test samples were used as controls. After incubation for 21 hours, 25 µl of MTT solution was added to each well. After an additional three hours of incubation, the experiment was terminated by addition of a solution of 10% sodium dodecyl sulfate in 0.01 N HCl. Following overnight incubation at 37°C to solubilize the formazane crystals, optical density was determined at 570-650 nm in microplate reader. The following formula was used to calculate % of toxicity:

$$\% \text{ of toxicity} = (1 - \text{sample OD} / \text{control OD}) \times 100$$

Samples were defined as cytotoxic when toxicity was greater than 25% in the assay system used. The results are shown in Tables I and II above.

Example 14

Treatment of Heart Transplant Rejection

Heterotopic whole heart transplantation was performed according to the standard method (Ono and Lindsey, 1969). The donors (Brown Norway rats, 200-255g, Charles River, Wilmington, MA) and recipients (Adult male Lewis rats, 225-275g, Charles River) were anesthetized with sodium pentobarbital (40 mg/kg). Following adequate donor anticoagulation using heparin, the heart graft was removed and stored at 4°C in PhysioSol Irrigation Solution (Abbott Laboratories, N. Chicago, IL). The ascending aorta and pulmonary artery were transected, and the vena cava and pulmonary veins were ligated. The recipient abdominal aorta and inferior vena cava were exposed through a median abdominal incision. The donor heart aorta and pulmonary artery were anastomosed end-to-side to recipient's infrarenal abdominal aorta and inferior vena cava, respectively, with running 8-0 monofilament nylon suture (Ethilon, Inc., Somerville, NJ). Because of the functional properties of the aortic valve, blood did not enter the left ventricle but

rather flowed through the coronary arteries to the right atrium, pulmonary artery and the recipient vena cava. The cold ischemic time of all the cardiac grafts was less than 45 minutes. Graft heart-beat was monitored by abdominal palpation. The period of functional graft survival was measured as the number of days during which cardiac graft contractions could be detected by abdominal palpation. Results were confirmed by direct visualization at laparotomy.

Heart transplant recipient animals prepared as described above (3-5 animals/group) were treated with (i) control solution (5% ethanol, 10 ml/kg), (ii) YM-273 at 0.10 mg/kg and 0.40 mg/kg (Fig. 5A), (iii) YM-274 at 0.084 and 0.33 mg/kg (Fig. 5B), and (iv) and T10 (triptolide) 0.25 mg/kg (Fig. 6). In addition to showing the results for T10, Fig. 6 repeats the results from Figs. 5A-5B for YM-273 and YM-274 at 0.40 and 0.33 mg/kg, respectively, such that their concentrations were equimolar relative to T10. All compounds were administered intraperitoneally. Treatment started on the day prior to surgery and continuing daily until postoperative day 14, or until the end of allograft survival. Results are shown in Figs. 5A-5B and 6.

Example 15

Effect of Triptolide Succinate on Fertility in Male Mice

Triptolide succinate (YM-262) was tested for fertility control in male BDF1 mice, using a cycle of daily administration for 5 days followed by a two day cessation of treatment. The cycle was repeated for 5 weeks, with dosing on days 0-4, 7-11, 14-18, 21-25 and 28-32 before evaluation of physiological effects and reproductive performance.

A dosing solution of YM-262 was prepared once a week from a stock solution in saline at 1.0 mg/ml and stored at 4°C. Mice were treated intraperitoneally (IP) with the saline vehicle or with YM-262 at 0.04 or 0.13 mg/kg/day in saline, using a 1 ml sterile disposable plastic syringe and a 25 or 26 gauge sterile hypodermic needle. The compound was administered in a volume corresponding to 0.1 ml per 10 g of mouse body weight.

A. Effect on Testis Weight

On day 32, five to six mice from each dosing group were sacrificed, and the testes were removed and stored in formalin for histological analysis and weight determination. The average of both testes was calculated for each mouse, and the mean and standard error of the mean (S.E.) were determined. These data are shown in Fig. 7.

In comparison to the saline-treated control group, a modest (approximately 13%) decrease in testis weight was observed with the mice receiving 0.1 mg/kg/day of YM-262.

B. Effect on Fertility

Fertility was tested with five additional male mice in each treatment group. Males were housed with two females per male on day 32, after which time no further YM-262 treatments were given. The first female in each cage to deliver a litter was removed to a separate cage with her
5 litter, and the remaining female was housed with the male. All male mice sired litters with both of the cohabiting females. Offspring were enumerated as to birthdate, sex and coat color.

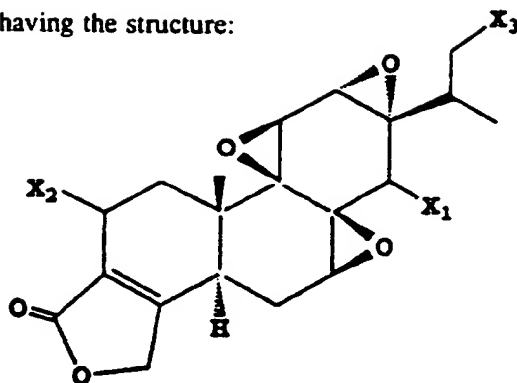
The time from beginning of cohabitation to birth of the litters was evaluated and is shown in Fig. 8. Again, the mean and standard error of the mean (S.E.) are shown. The saline-treated (control) group produced the first litter after a mean of 24 days. The value for the group receiving
10 YM-262 at 0.03 mg/kg/day was 28 days, a 17% delay. However, the time from cohabitation to birth was 45 days for the group receiving YM-262 at 0.1 mg/kg/day, an 88% increase compared to the control group.

These results indicate that YM-262 treatment at 0.1 mg/kg/day over a period of 32 days delayed the recovery of fertility after termination of therapy by 21 days. Furthermore, the control
15 of fertility was reversible, and it is expected that continued treatment would maintain the demonstrated control of fertility.

While the invention has been described with reference to specific methods and embodiments, it will be appreciated that various modifications may be made without departing from the invention.

IT IS CLAIMED:

1. A compound having the structure:



wherein

- 5 X^1 is OH or OR^1 , and X^2 and X^3 are independently OH, OR^1 or H, with the proviso that at least one of X^1 , X^2 and X^3 is OR^1 , and at least one of X^2 and X^3 is H; and
 R^1 is $-C(O)-Y-Z$, wherein
 Y is a branched or unbranched C_1-C_6 alkyl or alkenyl chain; and
 Z is $COOR^2$, NR^3R^3' , or $^+NR^4R^4'R^{4''}$, where
 10 R^2 is a cation;
 R^3 and R^3' are independently H or branched or unbranched C_1-C_6 alkyl, hydroxyalkyl, or alkoxyalkyl, or R^3 and R^3' taken together form a 5- to 7-member heterocyclic ring whose ring atoms are selected from the group consisting of carbon, nitrogen, oxygen and sulfur, wherein said ring atoms include 2 to 6 carbon atoms, one or more nitrogen atoms, and optionally one or more
 15 oxygen or sulfur atoms, and said ring is unsubstituted or is substituted with one or more groups selected from R^5 , OR^5 , NR^5R^6 , SR^5 , NO_2 , CN, $C(O)R^5$, $C(O)NR^5R^6$, $OC(O)R^5$, $OC(O)NR^5R^6$, and halogen, where R^5 and R^6 are independently hydrogen, lower alkyl or lower alkenyl; and
 R^4 , R^4' , and R^4'' are independently branched or unbranched C_1-C_6 alkyl, hydroxyalkyl, or alkoxyalkyl.
- 20 2. The compound of claim 1, wherein $X^2 = X^3 = H$.
3. The compound of claim 1, where $X^2 = H$.
4. The compound of claim 1, where $X^3 = H$.
5. The compound of any of claims 1-4, wherein Z is COOH.

6. The compound of any of claims 1-4, wherein Z is COOR², and R² is a metal ion.
7. The compound of claim 6, wherein the metal ion is Na⁺ or K⁺.
8. The compound of any of claims 1-4, wherein Z is COOR², and R² is an organic amine.
9. The compound of claim 8, wherein the organic amine is lysine, triethylamine, or tris
5 (hydroxymethyl)aminomethane.
10. The compound of any of claims 1-4, wherein Z is NR³R^{3'} and R³ and R^{3'} are independently H or branched or unbranched C₁-C₆ alkyl.
11. The compound of any of claims 1-4, wherein Z is NR³R^{3'} in protonated form or ⁺NR⁴R^{4'}R^{4''}, and said compound includes an anionic counterion.
- 10 12. The compound of claim 11, wherein the anionic counterion is chloride or bromide.
13. The compound of claim 11, wherein the anionic counterion contains a carboxylate ion, sulfonate ion, or sulfate ion.
14. The compound of claim 11, wherein the anionic counterion is acetate, oxalate, maleate, fumarate, methanesulfonate, or toluenesulfonate.
- 15 15. The compound of any of claims 1-4, wherein Y is a C₁-C₄ alkyl chain, and Z is COOR².
16. The compound of claim 15, wherein R² is Na⁺.
17. The compound of claim 16, wherein R² is tris(hydroxymethyl)ammonium methane or lysine.
18. The compound of any of claims 1-4, wherein Y is a C₁-C₄ alkyl chain, and Z is dimethyl amino.
- 20 19. The compound of any of claims 1-4, wherein Y is a C₁-C₄ alkyl chain, and Z is N-morpholino.

20. A pharmaceutical composition for immunosuppressive treatment, comprising a compound in accordance with any of claims 1-19 in a pharmaceutically acceptable vehicle.
21. A pharmaceutical composition for reducing male fertility, comprising a compound in accordance with any of claims 1-19 in a pharmaceutically acceptable vehicle.
- 5 22. A pharmaceutical composition as in claim 20 or 21, wherein the vehicle is an aqueous carrier.
23. A method of effecting immunosuppression, comprising administering to a subject in need of such treatment, a compound in accordance with any of claims 1-19, in a pharmaceutically acceptable vehicle.
- 10 24. The method of claim 23, wherein said immunosuppression comprises inhibition of transplant rejection.
- ✓ 25. The method of claim 23, wherein said immunosuppression comprises inhibition of graft-versus-host disease.
- ✓ 26. The method of claim 23, wherein said immunosuppression comprises treatment of an auto-immune disease.
- 15 27. The method of claim 26, wherein said autoimmune disease is rheumatoid arthritis.
28. A method of reducing fertility in a male mammal, comprising administering to such a subject in need of such treatment, a compound in accordance with any of claims 1-19, in a pharmaceutically acceptable vehicle.
- 20 29. A method of treating asthma, comprising administering to a subject in need of such treatment, a compound in accordance with any of claims 1-19, in a pharmaceutically acceptable vehicle.
30. The method of claim 29, wherein the compound is administered via inhalation.

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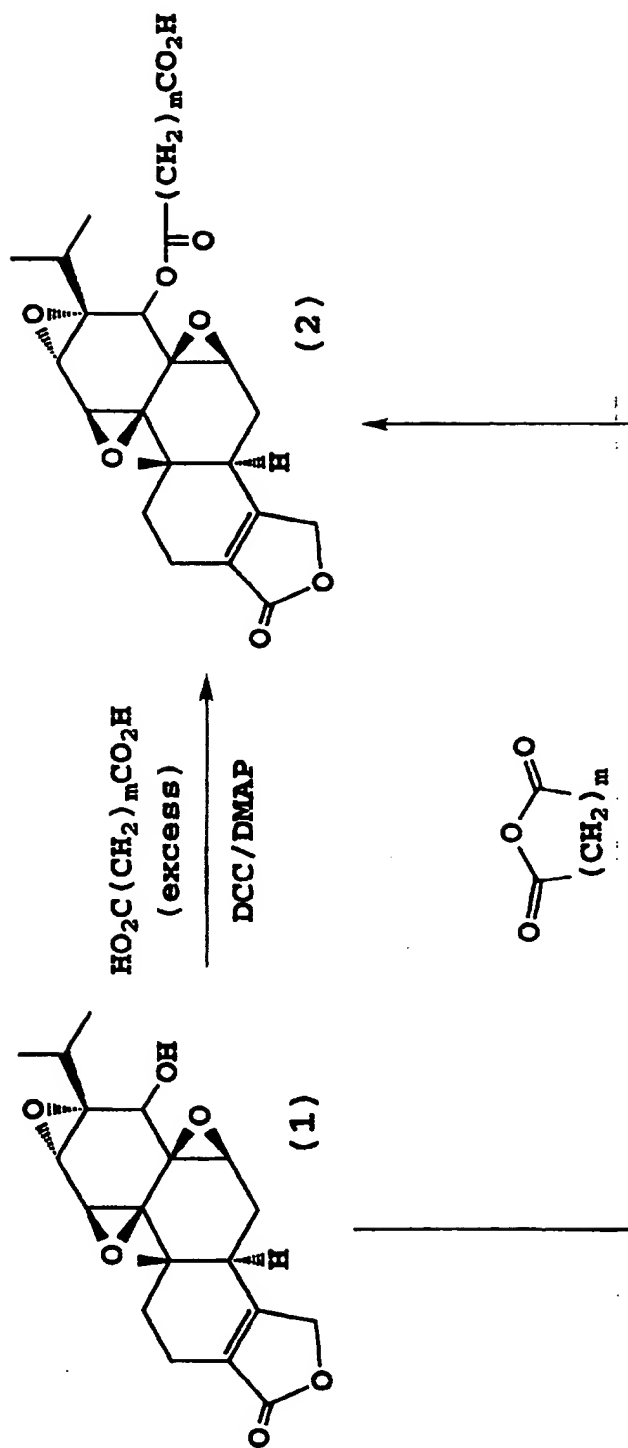


Fig. 1

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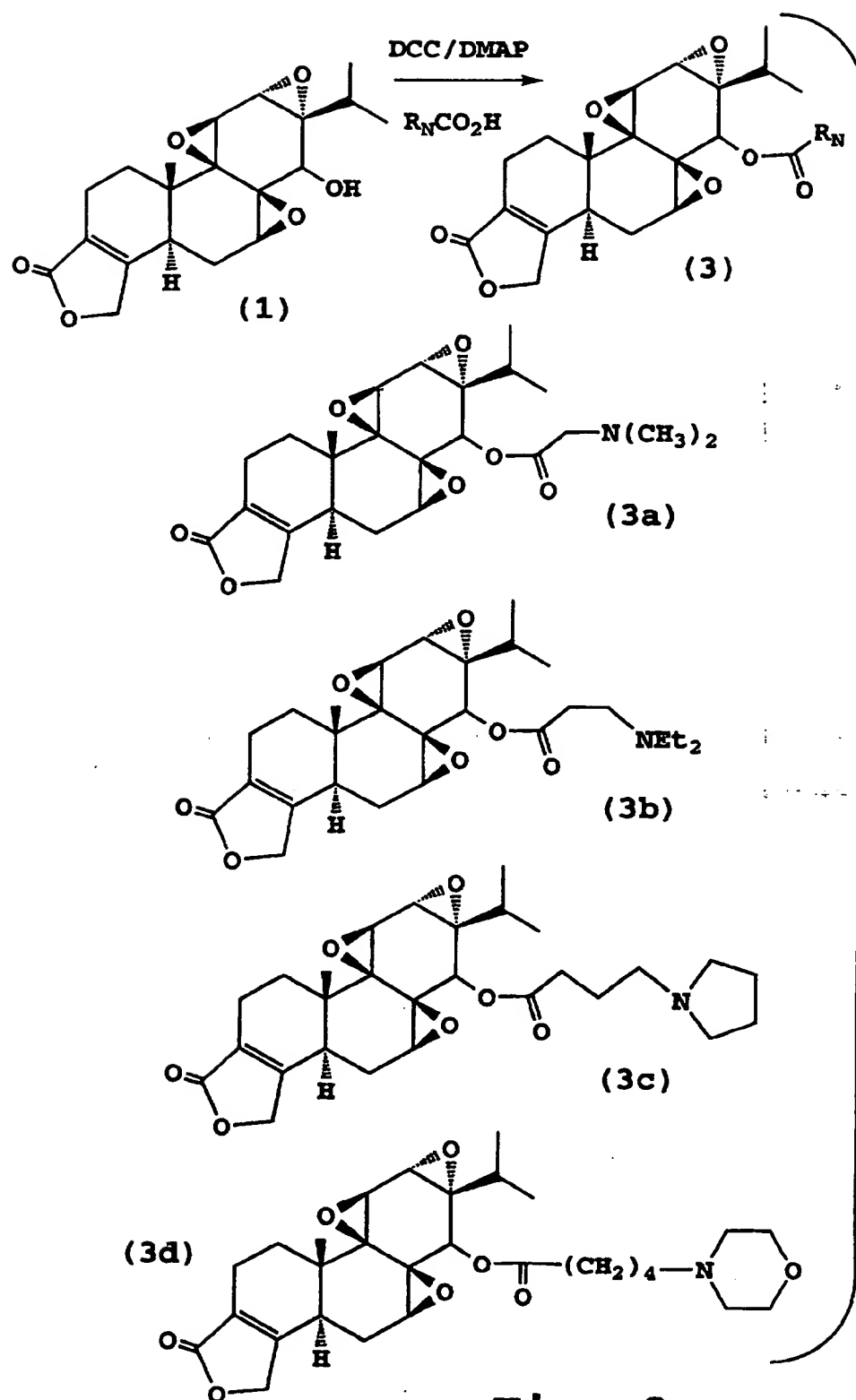


Fig. 2

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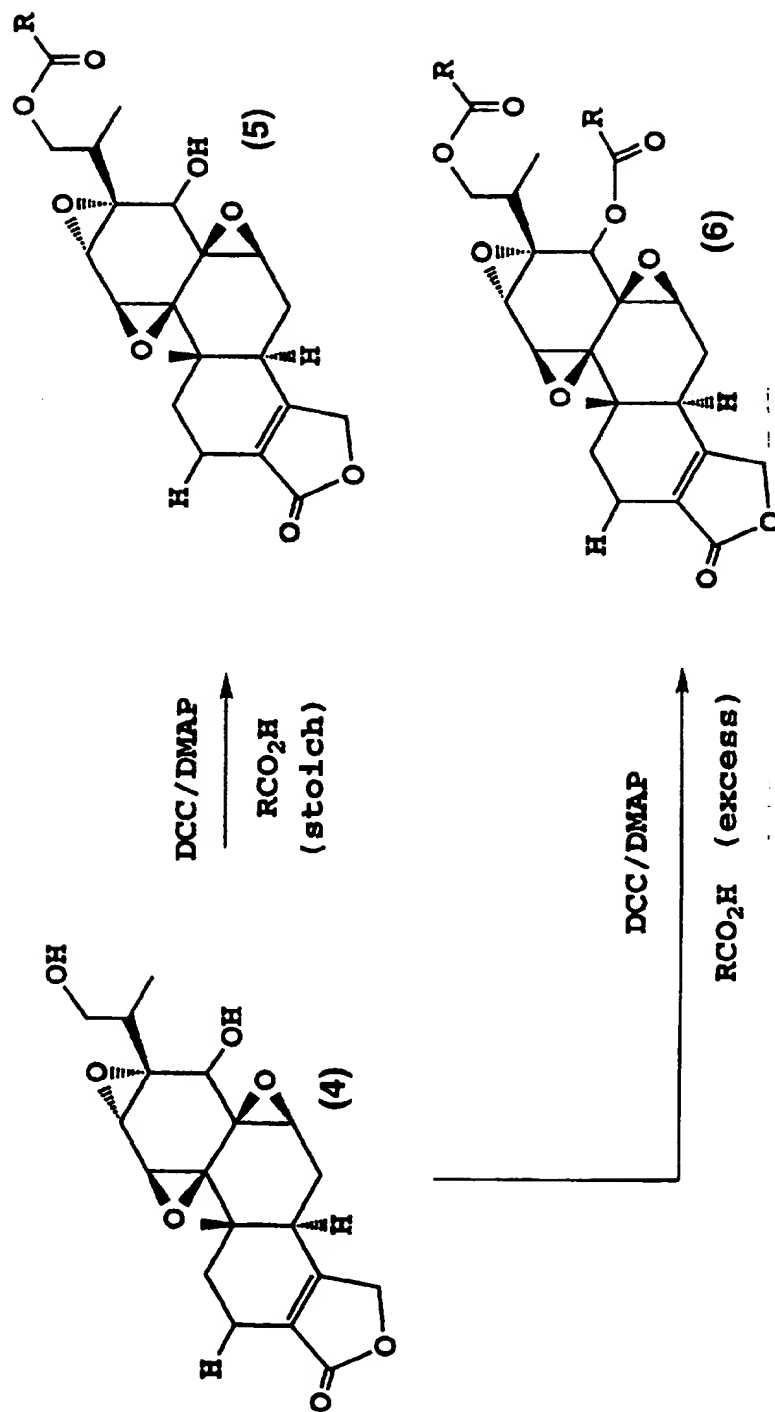


Fig. 3

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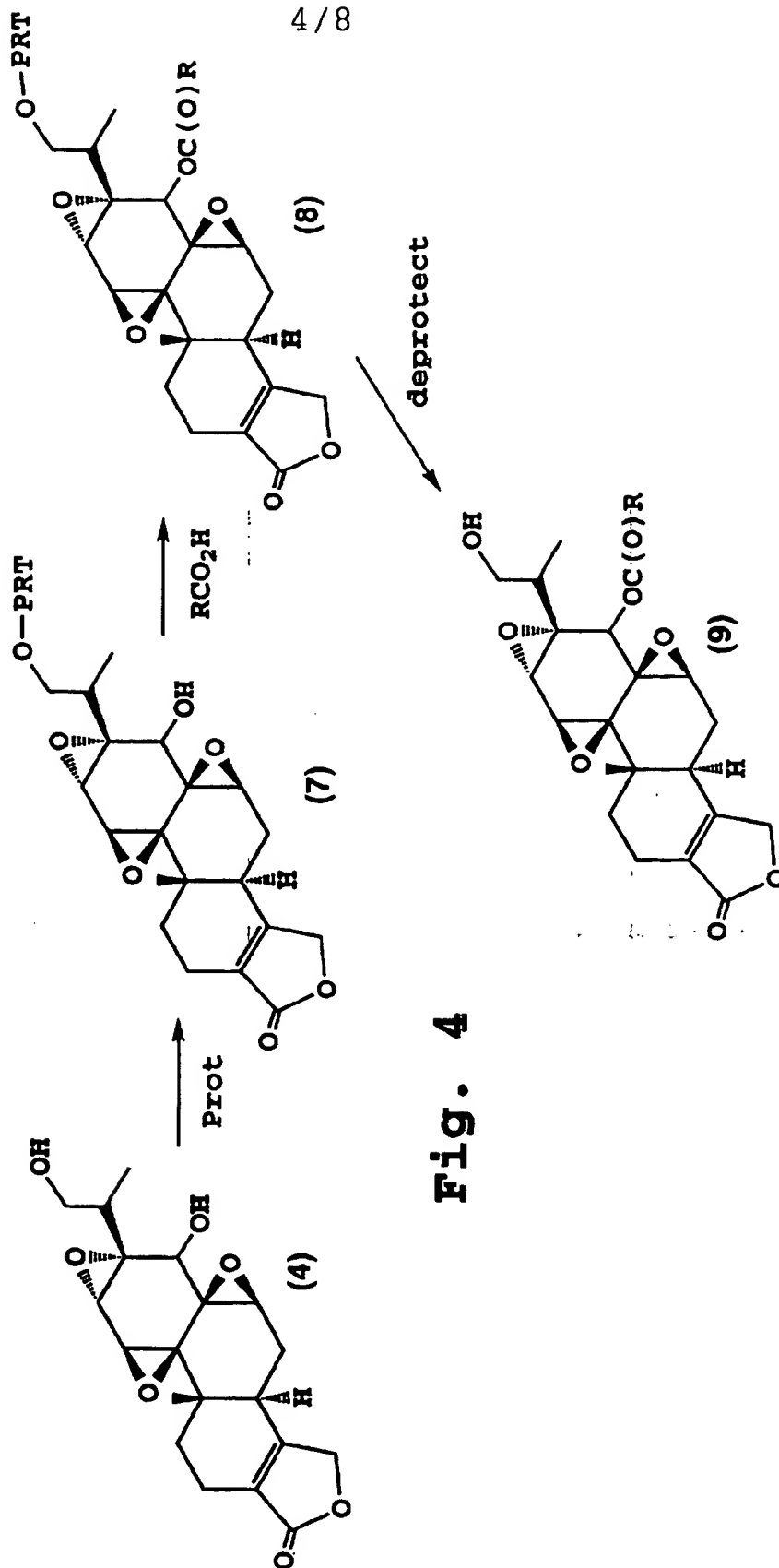
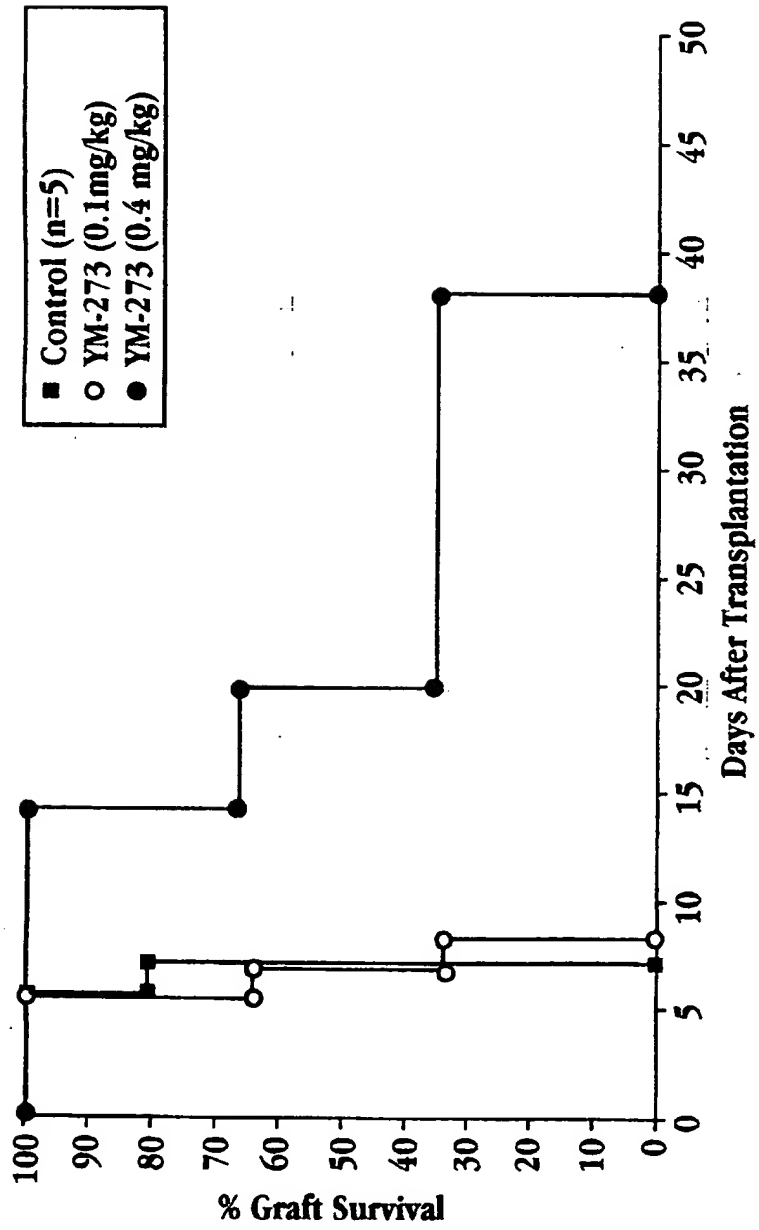
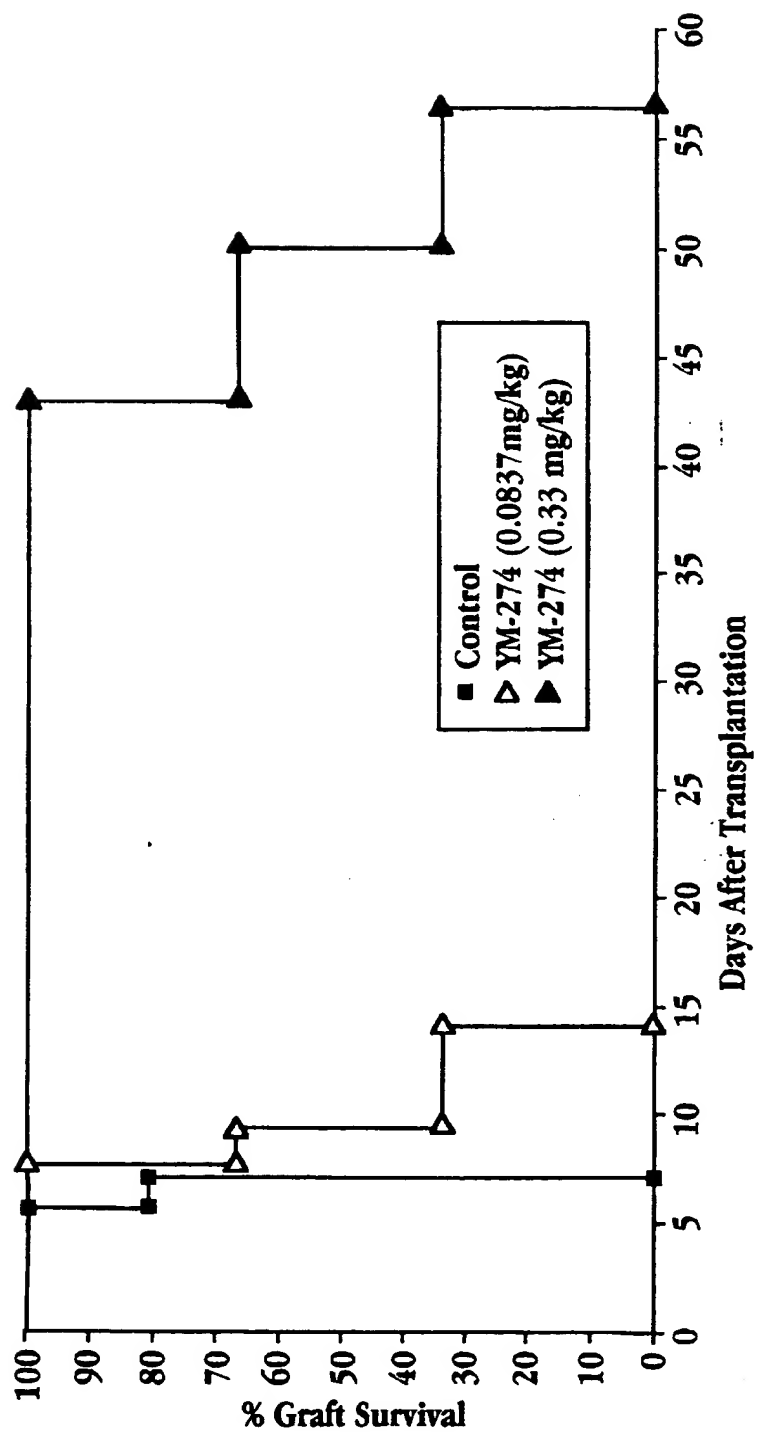


Fig. 4

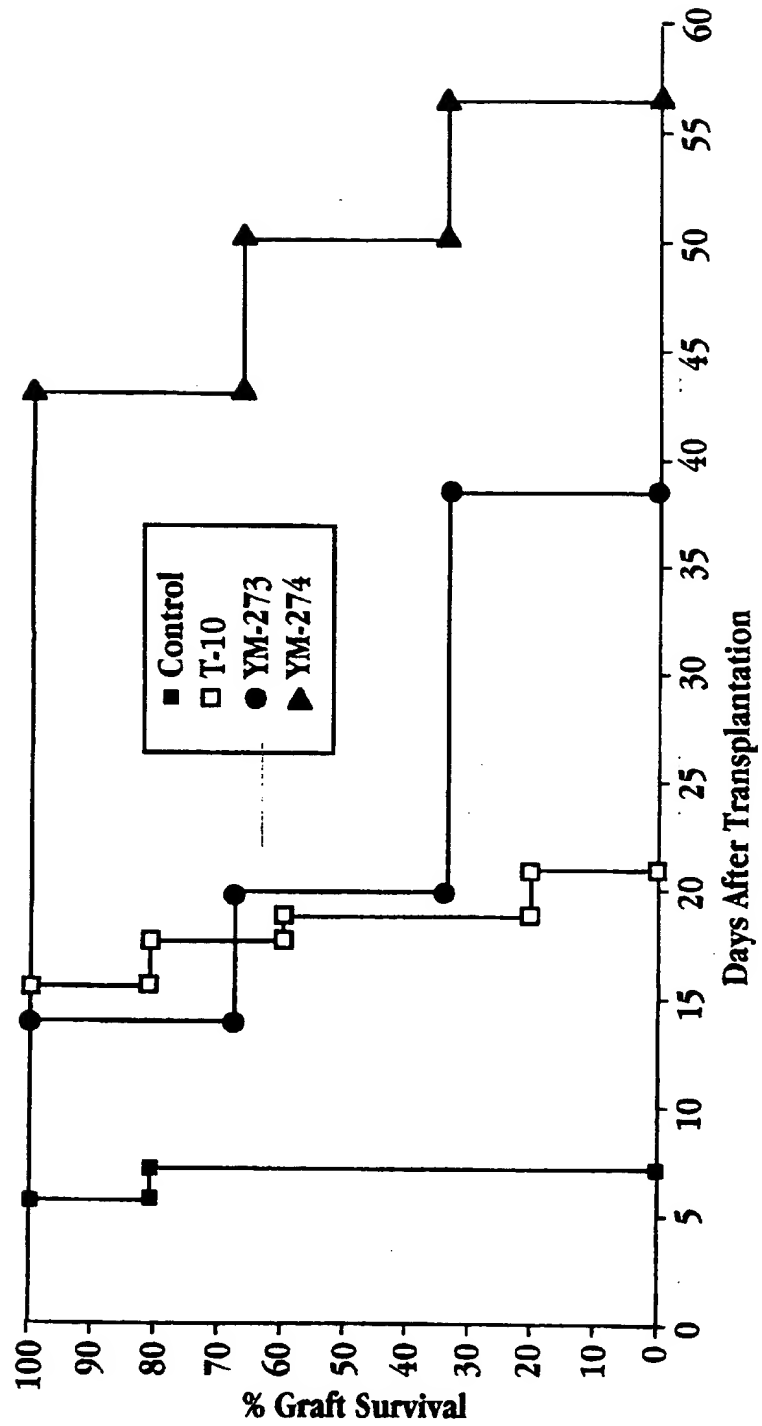
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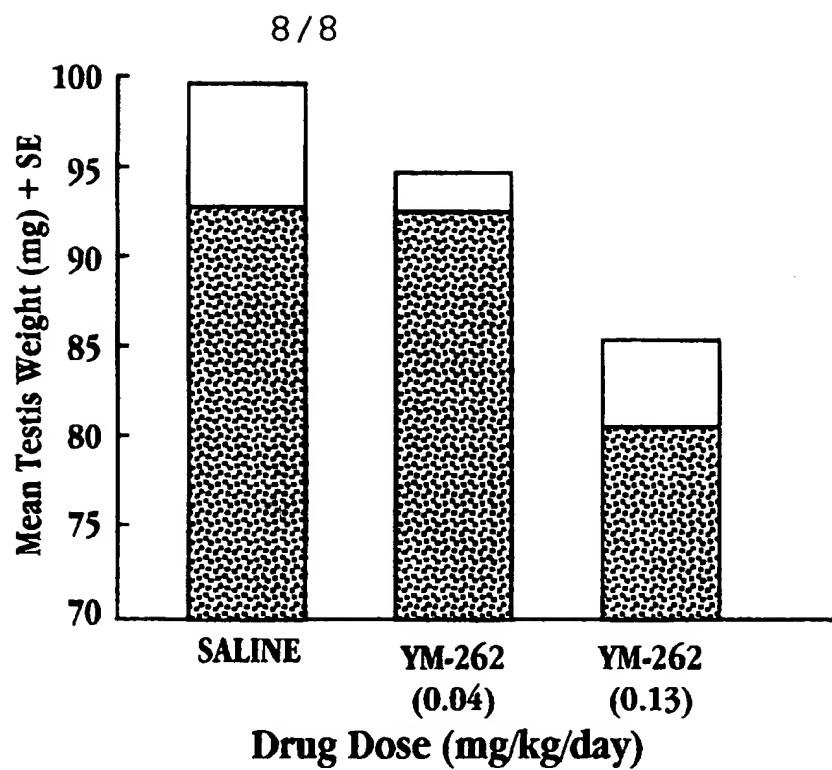
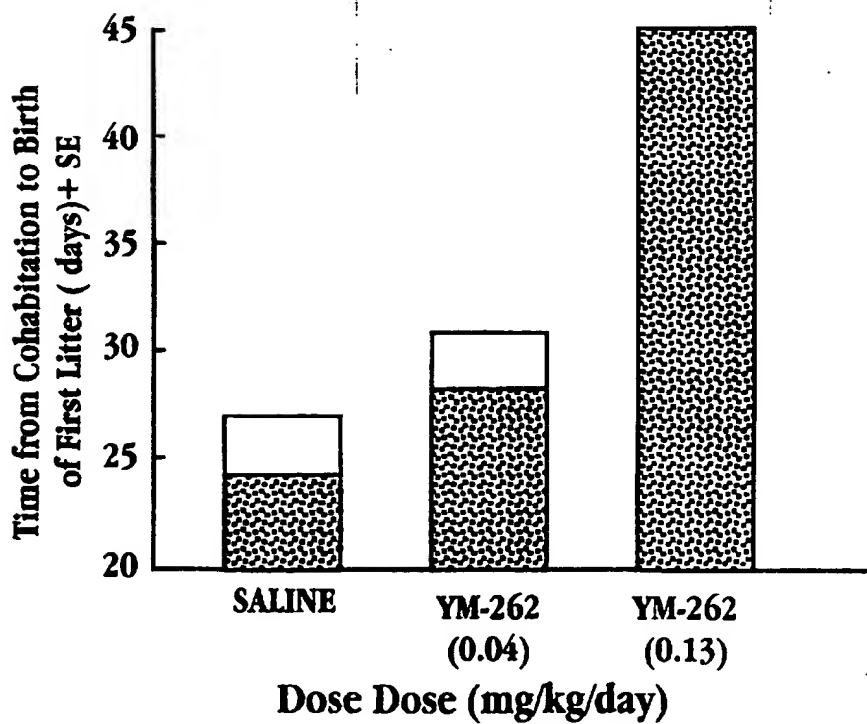
**Fig. 5A**

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**Fig. 5B**

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**Fig. 6**

**Fig. 7****Fig. 8**

INTERNATIONAL SEARCH REPORT

International Application No.

PCT/US 97/03202

A. CLASSIFICATION OF SUBJECT MATTER
 IPC 6 C07D493/22 A61K31/34 //(C07D493/22,307:00,303:00,303:00,
 303:00)

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 6 C07D A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	CHEMICAL ABSTRACTS, vol. 117, no. 9, 31 August 1992 Columbus, Ohio, US; abstract no. 83085a, ZHENG ET AL: "Screening of active antiinflammatory ..." XP002032698 &Zhongguo Yixue Kexueyuan Xuebao 1991, 13(6), 391-397 see abstract --- -/-	1-30

☒ Further documents are listed in the continuation of box C.

☒ Patent family members are listed in annex.

* Special categories of cited documents:

- "A" document defining the general state of the art which is not considered to be of particular relevance
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Date of the actual completion of the international search

30 June 1997

Date of mailing of the international search report

18.07.97

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INTERNATIONAL SEARCH REPORT

International Application No.

PCT/US 97/03202

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	CHEMICAL ABSTRACTS, vol. 116, no. 14, 6 April 1992 Columbus, Ohio, US; abstract no. 136234a, MA ET AL.: "Isolation of 17-hydroxytriptolide and analogs as drugs" XP002032699 & CN-A-1052859 see abstract	1-30
Y	US 5 430 054 A (QIAN SHAO-ZHEN ET AL) 4 July 1995 see the whole document	1-30
Y	US 5 294 443 A (LIPSKY PETER E ET AL) 15 March 1994 see figures 13-17	1-30
Y	EP 0 156 643 A (UPJOHN CO) 2 October 1985 see the whole document	1-30

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Intern. Application No

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